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Gonococcal ribosomes as skin test antigens
II. Precision of the method, attempts to identify the ribosomal antigen, and correlation with the macrophage migration inhibition test

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Summary

Gonococcal crude ribosome preparation was found to be both specific and sensitive when used to elicit delayed hypersensitivity reactions in sensitized guinea-pigs. Gonococcal crude ribosomes possess the ability to react with humoral antibody in sensitized animals; thus, ribosomes may be of value as an antigen in a sero-diagnostic test for gonorrhoea. Ribosomal protein or RNA, or gonococcal cell components including protoplasm, cell walls, and pilin, were less reactive than intact ribosomes. The ribosome preparation was used in an *in vitro* correlate of delayed hypersensitivity, suggesting that the human immune response in gonorrhoea might be studied using the crude ribosome in *in vitro* experiments with human peripheral lymphocytes.

Introduction

In the preceding paper (Koostra, Judd, and Baker, 1976), we reported the finding that ribosomes of *Neisseria gonorrhoeae* were reactive in skin testing of guinea-pigs which had previously been sensitized with killed cells of *N. gonorrhoeae*. In this paper we report on the precision of the method when delayed hypersensitivity reactions are the criterion employed. An earlier and more rapid skin test reaction of the Arthus type was also noted and is reported. Since ribosomes are large and complex particles, attempts were made to isolate their component RNA and proteins and additional potential cellular antigens to test their skin test reactivity in sensitized animals. Finally, we utilized the macrophage migration inhibition test (MIF), using peritoneal macrophages from sensitized guinea pigs, as an *in vitro* correlate of skin test reactivity.

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Material and methods

Sensitization of guinea-pigs, preparation of crude ribosomes, and skin testing were performed as previously described (Koostra and others, 1976).

BREAKDOWN OF GONOCOCCAL RIBOSOMES INTO 50S–30S SUBFRACTION POOL

The 50S–30S pool was prepared by re-suspending a crude ribosome preparation in 50S buffer. No attempt was made to isolate specific subunits from this preparation.

Purity of the ribosomal preparations was assayed using the ratio of absorption at 260 and 280 nm. In a pure ribosome preparation the ratio should be approximately 1·9 (Thompson and Snyder, 1971). All ribosome preparations used had a 260/280 nm ratio between 1·85 and 1·95.

EXTRACTION OF PROTEIN FROM GONOCOCCAL CRUDE RIBOSOMES

Protein from the crude ribosome preparation was isolated by the 6·0 M LiCl–8·0 M urea method of Spitnik-Elson (1965). A second method was the 2-chloroethanol method described by Fogel and Sypherd (1968). Protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

EXTRACTION OF RNA FROM GONOCOCCAL CRUDE RIBOSOMES

Ribosomal RNA was extracted with 90 per cent. phenol as described by Kirby (1958).

ISOLATION OF GONOCOCCAL CELL WALLS

The pellets from the protoplasm isolation centrifugation were washed twice in saline, combined, lyophilized, and stored at —80°C. until needed.

ISOLATION OF GONOCOCCAL PILIN

Pilin, the protein of gonococcal pili, was prepared as described by Buchanan, Swanson, Holmes, Kraus, and Gotschlich (1973) from cells of T1 and T2 colony types (Kellogg, Peacock, Deacon, Brown, and Pirkle, 1963) grown in 15 litre quantities in a fermenter in Thayer-Martin medium without inhibitors (Thayer and Martin, 1966).

SKIN TESTING TO DETERMINE SENSITIVITY OF CRUDE RIBOSOMES

Nineteen guinea-pigs were sensitized as described with cold-killed *N. gonorrhoeae*; gonococcal cell walls, protoplasm, crude ribosomes, and a 50S–30S subfraction pool were diluted to the following concentrations: 10·0, 5·0, 1·0, 0·5, 0·25, and 0·125 μg./0·1 ml. 0·1 ml. of each dilution was injected intradermally. The reactions were observed 4 hrs after injection for an immediate (Arthus) reaction and 24 hrs after injection for the delayed hypersensitivity reaction.

SKIN TESTING TO DETERMINE SPECIFICITY OF CRUDE RIBOSOMES

This series of skin tests was designed to determine the specificity of the crude ribosome preparation. Crude ribosomes from both gonococci and meningococci, as well as gonococcal whole cells, cell components, and ribosomal components were used to skin test animals sensitized with either *N. gonorrhoeae* or *N. meningitidis*.

Ten animals were sensitized with cold-killed *N. gonorrhoeae* and ten with heat-killed *N. meningitidis*. As already described (Koostra and others, 1976), all twenty animals were skin tested with dilutions of meningococcal and gonococcal crude ribosome preparations. One week later eight animals from each group were skin tested with dilutions of meningococcal and gonococcal crude ribosomes, gonococcal crude ribosomal protein, and gonococcal protoplasm. The following week, seven animals were then re-tested with gonococcal and meningococcal crude ribosome dilutions. After an additional period of 14 days, five animals from each group were then skin tested with gonococcal and meningococcal whole cells, and gonococcal crude ribosomes RNA, crude ribosome protein, and pilin.

In vitro CORRELATE OF DELAYED HYPERSENSITIVITY. MACROPHAGE MIGRATION INHIBITION TEST (MIF)

To determine if the crude ribosome could be used in an *in vitro* test of delayed hypersensitivity, the method described by George and Vaughn (1962) and by David, Al-Askari, Lawrence, and Thomas (1964) and David, Lawrence, and Thomas (1964) for macrophage migration inhibition was employed, using sterile Sykes-Moore chambers (2 tubes/chamber, each in triplicate). They were sealed and filled with the following:

Control

One-third of the chambers: Eagle's Modified Minimal Essential Media (EMMEM) (Gibco) containing 10 per cent. fetal calf serum (FCS) (Gibco) plus 100 μg. streptomycin and 100 units penicillin/ml. plus $MgCl_2$ (0·01 M).

GONOCOCCAL RIBOSOMES

One third of the chambers: EMMEM containing 10 per cent. FCS plus 100 μg. streptomycin and 100 units penicillin/ml. plus $MgCl_2$ (0·01 M) plus 0·3 μg. gonococcal crude ribosomes.

MENINGOCOCCAL RIBOSOMES

EMMEM containing 10 per cent. FCS plus 100 μg. streptomycin and 100 units penicillin/ml. plus $MgCl_2$ (0·01 M) plus 0·3 μg. meningococcal crude ribosomes.

The chambers were incubated at 37°C. for 24 hrs. Areas of migration were determined by projecting the images of migration on to high quality bond paper using a photographic enlarger. The outlines of the images were drawn on the paper and the outlined regions were cut out. These were then weighed and compared with weights of known areas to determine the area of migration.

Results

The delayed hypersensitivity reactions provoked by gonococcal crude ribosomes as previously reported (Koostra and others, 1976) were compared to those provoked by gonococcal protoplasm, cell walls, and 50S-30S ribosomal subunit pool. The skin injection sites were observed for an immediate (Arthus) reaction at 4 hrs after antigen administration as well as for the delayed hypersensitivity reaction 24 hrs after injection.

The results of these tests appear in Table I. Examination showed that an immediate reaction did occur, indicating a humoral response to the antigens, and that this response could be correlated with the delayed reaction. Crude ribosomes were most reactive, while cell walls and protoplasm gave relatively

TABLE I *Immediate* and delayed hypersensitivity reactions† elicited in guinea-pigs sensitized with* N. gonorrhoeae *and skin tested with gonococcal crude ribosomes, cell walls, protoplasm, and 50S-30S ribosomal pool*

STD₅₀[c] (μg.)	*Antigen used to skin test*		*Amount of antigen provoking reaction* (μg.)				
			10·0	5·0	1·0	0·5	0·25
0·32	Crude ribosomes	Immediate	233·8[a]	146·3	80·9	54·7	41·4
		Delayed	19/19[b]	19/19	19/19	17/19	7/19
8·20	Cell walls	Immediate	76·0	60·8	56·2	19·0	10·9
		Delayed	13/19	7/19	0/19	0/19	0/19
7·50	Protoplasm	Immediate	112·3	59·2	43·5	3·6	1·2
		Delayed	12/19	7/19	2/19	0/19	0/19
0·98	50S–30S pool	Immediate	148·7	62·4	59·2	23·0	41·4
		Delayed	13/19	13/19	10/19	4/19	0/19

*Reaction measured 4 hrs after antigen injection (Arthus reaction)
†24-hr reaction greater than 10 × 10 × 0·4 mm. (15 mm.³)
[a]Mean area of lesion in mm.²
[b]Numerator—No. of positives. Denominator—No. of animals tested
[c]Skin test dose 50

weak reactions. The 50S-30S subunit pool possessed less activity than the crude ribosome preparation. It should be noted that, while the immediate reactions occurred with all the antigens at the lowest (0·25 μg.) dilution, no attempt was made to define a positive Arthus reaction. The delayed reactions also occurred at very low concentrations of antigen, but these were not great enough to meet the definition of a positive skin test (15 mm^3). Histological sections of the immediate and delayed lesions were prepared and photomicrographs were made (Judd and Koostra, unpublished data).

Table II displays the results of skin testing gonococcal- and meningococcal-sensitive animals with gonococcal and meningococcal crude ribosomes. As in the first series of skin tests, the gonococcal crude ribosomes manifested specificity due to their sensitivity. The meningococcal crude ribosomes demonstrated similar specificity and sensitivity in animals sensitized with homologous organisms. The STD_{50} showed that the results were consistent from test to test and that the crude ribosomes were from six to twelve times as potent in animals sensitized with homologous organisms as in animals sensitized with heterologous organisms.

Table III displays the results of skin testing in animals sensitized with either *N. gonorrhoeae* or *N. meningitidis*, using two preparations of gonococcal ribosomal protein and two preparations of gonococcal ribosomal RNA as skin test antigens. In all

TABLE II *Delayed hypersensitivity* reactions elicited in guinea-pigs sensitized with either* N. gonorrhoeae *or* N. meningitidis *and skin tested with gonococcal and meningococcal crude ribosomes*

Guinea-pigs	STD_{50}[c] (μg.)	*Antigen used to skin test*	*Amount of antigen provoking reaction (μg.)*					
			10·0	5·0	1·0	0·5	0·25	0·125
Gonococcal-sensitized	0·34	Gonococcal crude ribosomes	10/10[a]	10/10	10/10	10/10	2/10	0/10
	0·25		—[b]	8/8	8/8	8/8	4/8	0/8
	0·29		—	7/7	7/7	6/7	3/7	0/7
	4·60	Meningococcal crude ribosomes	8/10	6/10	0/10	0/10	0/10	—
	2·60		—	4/8	1/8	0/8	0/8	0/8
	4·53		—	6/7	2/7	0/7	0/7	0/7
Meningococcal-sensitized	3·36	Gonococcal crude ribosomes	6/10	6/10	2/10	0/10	0/10	0/10
	2·60		—	5/8	3/8	1/8	0/8	0/8
	1·96		—	6/7	3/7	0/7	0/7	0/7
	0·43	Meningococcal crude ribosomes	10/10	10/10	8/10	8/10	0/10	0/10
	0·32		—	7/7	7/7	5/7	3/7	0/7

*24-hr reactions greater than 10 × 10 × 0·4 mm. (15 $mm.^3$)
[a]Numerator—No. of animals positive. Denominator—No. of animals tested
[b]This dilution not tested
[c]Skin test dose 50

TABLE III *Delayed hypersensitivity reactions* elicited in guinea-pigs sensitized with either* N. gonorrhoeae *or* N. meningitidis *and skin tested with two preparations of gonococcal crude ribosome protein and two preparations of gonococcal crude ribosome RNA*

Guinea-pigs	STD_{50}§ (μg.)	*Antigens used to skin test*	*Amount of antigen provoking the reaction (μg.)*					
			10·0	5·0	1·0	0·5	0·25	0·125
Gonococcal-sensitized	3·48	Gonococcal crude ribosome protein [a] [b]	—‡	5/8†	2/8	0/8	0/8	0/8
	3·92		5/5	3/5	1/5	0/5	0/5	—
	—	Gonococcal crude ribosome RNA [c] [d]	2/5	0/5	0/5	0/5	0/5	—
	—		0/2	0/2	0/2	0/2	0/2	—
Meningococcal-sensitized	—	Gonococcal crude ribosome protein [a] [b]	—	0/8	0/8	0/8	0/8	0/8
	—		1/5	0/5	0/5	0/5	0/5	—
	—	Gonococcal crude ribosome RNA [c] [d]	0/5	0/5	0/5	0/5	0/5	—
	—		0/1	0/1	0/1	0/1	0/1	—

[a]6·0 M LiCl–8·0 M urea preparation—Spitnik-Elson (1965)
[b]2-chloroethanol preparation—Fogel and Sypherd (1968)
[c]Residue of 2-chloroethanol extraction of protein
[d]Phenol extracted—Kirby (1958)
*24-hr reactions greater than 10 × 10 × 0·4 mm. (15 $mm.^3$)
†Numerator—No. of animals positive. Denominator—No. of animals tested
‡This dilution not tested
§Skin test dose 50

cases the protein elicited stronger reactions in the gonococcal-sensitive animals than in the meningococcal-sensitive guinea-pigs. In the dilutions tested, the phenol-extracted RNA failed to provoke delayed reactions in either group tested. The STD_{50} showed the protein preparations to be less than one-tenth as potent as the parent crude ribosome preparations.

Table IV presents the results of the skin testing with gonococcal whole cells, protoplasm, pilin, and meningococcal whole cells using gonococcal and meningococcal-sensitive guinea-pigs. The results showed none of these fractions to be as sensitive or specific as the crude ribosome preparations. Pilin, while displaying greater potency than most of the other antigens shown in these Tables, was still less reactive than the crude ribosome preparation.

The results of the macrophage migration inhibition test are given in the Figure. The gonococcal crude ribosomes (0·3 μg.) caused an 85 per cent. inhibition of macrophage migration of the gonococcal sensitive peritoneal exudate cells (PECs) compared to non-sensitized PECs. The meningococcal crude ribosomes showed a 60 per cent. inhibition of macrophage migration in meningococcal sensitive PECs compared to non-sensitized PECs, while inhibiting the gonococcal sensitive PECs less than 11 per cent. Migration of normal PECs was inhibited only 3 to 4 per cent. by the crude ribosome preparations. In a similar experiment at a concentration of 0·5 μg. ribosomes, a degree of non-specific toxicity to both sensitized and non-sensitized PECs was observed.

Discussion

The results of this study indicated that the gonococcal crude ribosome preparation possessed the sensitivity and specificity required to differentiate between guinea-pigs artificially sensitized with *N. gonorrhoeae* and animals sensitized with *N. meningitidis* when used as a skin test antigen in delayed hypersensitivity testing. The results were reproducible with good precision.

N. meningitidis has been shown by Kingsbury, Fanning, Johnson, and Brenner (1969), using DNA recombination studies, to be very closely related to *N. gonorrhoeae*. Also, *N. meningitidis* occurs endemically and might cross-react in any test designed to demonstrate gonorrhoea. Thus, the crude ribosome was able to differentiate between these two very closely related organisms in sensitized guinea-pigs.

In our experiments the crude ribosome preparation displayed better sensitivity and specificity than any of the other gonococcal cell fractions used as skin test antigens in delayed hypersensitivity testing. The immunology of the ribosome is only just beginning to be studied extensively. At present, many researchers are using ribosomes and ribosomal fractions in many different studies involving a variety of organisms. Data obtained thus far are often contradictory. Smith and Bigely (1972) have shown both ribosomal RNA and ribosomal protein from *Salmonella typhimurium* to be immunogenic. Venneman (1972) has reported *S. typhimurium* ribosomal protein to be non-immunogenic. Winston and Berry (1970) suggested that the immunogenicity of *Staphylococcus aureus* ribosomes was due to the ribosomal protein, but that the ribosomal RNA acted as an adjuvant. Thompson and Snyder (1971) noted that, in the *Diplococcus pneumoniae* system, both ribosomal RNA and protein were required for immunogenicity, but Youmans and Youmans (1966) demonstrated that the removal of the ribosomal RNA decreased the immunogenicity of the mycobacterial ribosome while removal of the protein did not. Baker and others (1972) have shown that the 30S ribosomal particle of *Mycobacterium bovis* (BCG) is three to four times

TABLE IV *Delayed hypersensitivity reactions* elicited in guinea-pigs sensitized with either* N. gonorrhoeae *or* N. meningitidis *and skin tested with gonococcal whole cells, protoplasm, and pilin, and meningococcal whole cells*

Guinea-pigs	*STD_{50}§ (μg.)*	*Antigens used to skin test*	*Amount of antigen provoking reaction (μg.)*					
			10·0	5·0	1·0	0·5	0·25	0·125
Gonococcal-sensitized	6·60	Gonococcal whole cells	4/5†	2/5	0/5	0/5	0/5	—‡
	—	Gonococcal protoplasm	—	3/8	0/8	0/8	0/8	0/8
	0·83	Gonococcal pilin	—	5/5	3/5	1/5	0/5	0/5
	8·50	Meningococcal whole cells	3/5	1/5	0/5	0/5	0/5	—
Meningococcal-sensitized	—	Gonococcal whole cells	2/5	2/5	0/5	0/5	0/5	—
	—	Gonococcal protoplasm	—	1/8	0/8	0/8	0/8	0/8
	1·64	Gonococcal pilin	—	5/5	2/5	0/5	0/5	0/5
	7·50	Meningococcal whole cells	4/5	1/5	1/5	0/5	0/5	—

*24-hr reactions greater than 10 × 10 × 0·4 mm (15 mm^3.)
†Numerator—No. of animals positive. Denominator—No. of animals tested
‡This dilution not tested
§Skin test dose 50

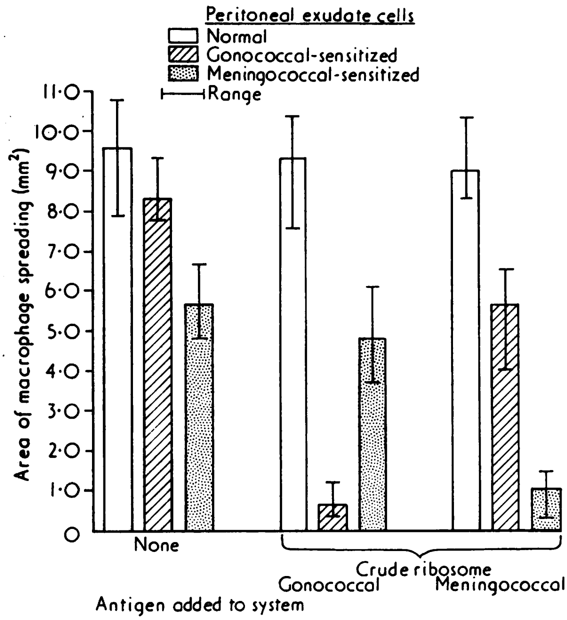

FIGURE *Results of macrophage migration inhibition test. Average area of spreading of macrophages of non-sensitized and* N. gonorrhoeae *and* N. meningitidis *sensitized guinea-pigs exposed to* 0·3 *µg. of either gonococcal or meningococcal crude ribosomes*

more potent a skin test antigen than either the 70S ribosome or the 50S subunit. Results of the present study show that this is not the case when ribosomes from *N. gonorrhoeae* are used as skin test antigens. Until more testing is done with gonococcal ribosomes, as well as with those from many other organisms, the exact nature of ribosomal immunology and immunogenicity will remain in question.

The immediate (Arthus) reactions observed 4 hrs after antigen injection paralleled the delayed reactions observed 24 hrs after antigen injection. The potential importance of these observations lies in the ability of the crude ribosomes to react with humoral antibody. It may therefore be of value as a potential antigen in a serodiagnostic test as well as in a skin test.

The macrophage migration inhibition tests showed the skin test reactions to be true cell-mediated delayed hypersensitivity responses, stimulated by ribosomes and mediated by lymphoid cells specifically sensitized with homologous organisms. Ribosomal antigens were not toxic for non-sensitized cells at the antigen concentration utilized.

The *in vitro* demonstration of cell-mediated immunity with the gonococcal and meningococcal crude ribosome preparations suggests the use of the macrophage migration test as a means of studying the human cell-mediated response to gonococcal infection, even though the guinea-pig model cannot be directly related to human infection. Peripheral lymphocytes from humans could be used as an *in vitro* test to determine the value of the gonococcal ribosome as a diagnostic test for human gonorrhoea.

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